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(54) **Method and reagents for in vitro oligonucleotide synthesis.**

(57) A method is provided for the high fidelity, rapid and economical in vitro synthesis of oligonucleotides. Nucleoside H-phosphonates are condensed in seriatim using a dehydrating agent to produce a poly (nucleoside H-phosphonate). The product is oxidized to yield the desired oligonucleotide. A novel reagent is provided for multiple nucleoside additions in single cycles.

EP 0 219 342 A2

Descript

METHOD AND REAGENTS FOR IN VITRO OLIGONUCLEOTIDE SYNTHESIS

This invention relates to the in vitrosynthesis of oligonucleotides. In particular it relates to the preparation of deoxyoligonucleotides for use in recombinant host-vector systems and as intermediates in the preparation of labelled oligonucleotide probes for diagnostic assays.

Oligonucleotides are presently prepared in vitro by one of two prevalent methods, the phosphoramidite method (for example see Adams et al.. 1983. "J. Amer. Chem. Soc." 105: 661 and Froehler et al., 1983 "Tetrahedron Lett." 24: 3171) or the phosphotriester route (German Offenlegungsshrift 2644432). Both methods, while efficient and widely accepted, employ large amounts of costly monomer. These methods are also complex. The phosphoramidite method requires an aqueous oxidation step after each condensation, while the triester method requires that the aborted subpopulation of oligonucleotides (those that have not had a monomer added during a cycle) be "capped" in a separate step in order to prevent further chain elongation of the aborted oligonucleotides in future cycles. It is an object of this invention to provide a method for the oligonucleotide synthesis that is more economical and reliable then the methods currently available.

It is known to prepare nucleoside phosphonates by condensation of nucleosides with phosphorous acid using aryl sulfonyl chloride (Sekine et al., 1979; "Tetrahedron Lett." 20: 1145), or carbodiimides (Schofield et al., 1961, "J. Am. Chem. Soc." page 2316). Hall et al. ([1957], "J. Chem. Soc." Nucleotides Part XLI, pp 3291-3296) further treated the nucleoside H-phosphonate to prepare the corresponding dinucleotide phosphonate using diphenylchorophosphate followed by oxidation to produce the corresponding dinucleotide phosphate. Thus, notwithstanding knowledge in the art relating to the preparation of dinucleotide phosphate since 1957, and the intervening development of two complex and costly methodologies for the in vitro preparation of oligonucleotides, the Hall et al. disclosure has remained unexploited for the preparation of oligonucleotides.

Garegg et al., "Chemica Scripta" 25: 280-282 (1985) found that 5'-O-dimethoxytritylthymidine 3'-hydrogen-phosphonate reacts rapidly with 3'-O-benzoylthymidine in the presence of an activator such as diphenylphosphorochloridate (also known as diphenylchlorophosphate), 2,4,6-triisopropylbenzenesulfonyl chloride or 2,4,6-triisopropylbenzenesulfonyl tetrazolide to form, in quantitative yield, a phosphonate diester bond. According to Garegg et al., the stability of the 3'-phosphonates when compared to the corresponding 3'-phosphoramidites, together with their comparable reactivity to phosphoramidites on activation, made them suitable as starting materials for oligonucleotide synthesis. The Garegg activating agents have been found by the inventors herein to be unsuitable for practical oligonucleotide synthesis because of the poor yields so obtained, a discovery that may help to explain the three decades that have passed without the art having harnessed phosphonate chemistry to the objective of oligonucleotide synthesis.

The synthesis of DNA containing internucleotide phosphate analogues is becoming a field of great interest. Miller and Ts'o have shown that methyl phosphonate analogues of DNA readily pass through cell membranes and inhibit protein synthesis, presumably by interfering with mRNA translation (Blake et al., Biochemistry 24 6139 (1985), Smith et al., Proc. Natl. Acad. Sci. 83, 2787 (1986)). Phosphoramidate analogues of dinucleotides are known to bind to complementary polynucleotides and can be used for the attachment of various ligands to DNA (Letsinger et al., Nucl. Acids Res. 14, 3487 (1986)). Oxidation of dinucleoside H-phosphonates in the presence of amines leads to the corresponding dinucleotide phosphoramidates (Nemer et al., Tetrahedron Lett. 21, 4149 (1980), Atherton et al., J. Chem. Soc. 660(1945)) in high yield. Methods are needed for the facile preparation of phosphoramidate, thiophosphate and phosphate triester analogues of oligonucleotides.

According to the present invention there is provided a method for synthesizing a polynucleotide which comprises (a) providing a nucleoside bound to a carrier, (b) condensing a blocked nucleoside H-phosphonate with the 5' hydroxyl of the carrier-bound nucleoside in the presence of a dehydrating agent, (c) removing the 5' blocking group of the nucleoside H-phosphonate of step b), (d) sequentially repeating steps b) and c) using selected nucleoside H-phosphonates until a polynucleotide H-phosphonate having greater than two nucleotides has been obtained, (e) oxidizing the polynucleotide H-phosphonate to form the corresponding polynucleotide, and (f) separating the polynucleotide from the carrier. The polynucleoside H-phosphonates are useful as starting materials for the novel synthesis of the phosphoramidate, thiophosphate and phosphate triester oligonucleotides.

The starting material for the practice of the inventive method comprise a carrier-bound nucleoside and blocked nucleoside H-phosphonate monomers. The 5' hydroxyl of the carrier bound nucleoside is not blocked. The nucleosides employed herein are the monomers of RNA or DNA or their derivatives. Exemplary ribose-containing nucleosides include adenosine, guanosine, inosine, cytidine, thymine ribonucleoside, and uridine. Deoxyribose-containing nucleosides include deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine. The first group of nucleosides are used in synthesizing mRNA or tRNA, the second group in preparing DNA.

The preferred blocked nucleoside H-phosphonate monomers are analogues of the nucleotides constituting nucleic acids. The blocking groups generally are triphenylmethyl ethers of the ribose or deoxyribose hydroxyl substituents. Their function is to restrict the condensation reaction to the 5' hydroxyl of the carrier-bound nucleoside or oligonucleotide. The preferred starting phosphonates for the preparation of oligodeoxynucleo-

tides and their correspo[illegible]g nucleosides, designated parenthetically, are 5[illegible]ethoxytrityl-3'-thymidine H-phosphonate (thymidine), 5' dimethoxytrityl-3'-[-N^6-benzoyldeoxyadenosine] H-phosphonate (deoxyadenosine), 5' dimethoxytrityl-3'-[N^4-benzoylcytidine] H-phosphonate (deoxycytidine), and 5' dimethoxytrityl-3'-[N^2-isobutyl deoxyguanosine] H-phosphonate (deoxyguanosine). The starting materials for the preparation of oligoribonucleotides are similar, for example for adenosine. 5' dimethoxytrityl-2'-dimethyl t-butyl silyl-[N^6-benzoyladenosine]-3' H-phosphonate. The starting phosphonates are prepared by methods known per se. For example, see Sekine et al., "Tetr. Lett." 16: 1711 (1973). Another method for preparing the starting phosphonates (which is preferred) employs tris (1, 2, 4-triazoyl) phosphite (TTP). TTP is conveniently generated in situ from phosphorus trichloride (PCl_3), 1, 2, 4-triazole and N-methyl morpholine in anhydrous methylene chloride (CH_2Cl_2).

Carriers are selected that are insoluble in the reagents used for the oligonucleotide synthesis, this being for convenience in recovering the polynucleotide after each condensation cycle. The carrier also is sufficiently porous to permit the facile entry of reactants and elution of product and reactants. A preferred carrier is controlled pore glass, although silica gel or polystyrene also are acceptable. Carrier-bound nucleosides are prepared by known procedures, for example Chow et al., " Nucl. Acids Res." 9: 2807 (1981).

Nucleoside H-phosphonates exist as an anion having a positively charged counterion. Triethyl ammonium (TEAH+ or 1,8 Diazabicyclo [5.4.0] undec-7ene-onium (DBUH+ are preferred counterions. The DBUH salts of phosphonates have increased stability in anhydrous solvents.

The counter bound nucleoside and the nucleoside 3' H-phosphonates are selected in seriatim so as to correspond to the predetermined sequence of the oligonucleotide to be synthesized, with the 3' nucleoside being carrier-bound. Oligonucleotide chain elongation proceeds in conformance with this predetermined sequence in a series of condensations, each one of which results in the addition of another nucleoside to the oligomer.

In the first of these condensations, the carrier-bound nucleoside is condensed with the second nucleoside in the oligonucleotide. The key reagent in this reaction as well as in the subsequent condensations is the dehydrating agent. Suitable dehydrating agent generally fall within the classes of phosphorylating agents or acylating agents and include isobutyl chloroformate, diphenyl chlorophosphate, organic acid anhydrides such as acetic anhydride, isobutyric anhydride and trimethylacetic anhydride, and organic acid halides such as pivaloyl chloride, pivaloyl bromide and benzoyl chloride. Diphenylchlorophosphate, carbodiimides, aryl sulfonyl chlorides and aryl sulfonyl tetrazolides in our experience result in extremely poor yields. The property of the acylating or phosphorylating agent that is most important for the purposes of preparing oligonucleotides is its ability to add a nucleoside to substantially all of the nascent carrier-bound chains at each cycle. If the additional nucleoside is not added to a subpopulation of chains, i.e. a cycle is aborted as to that subpopulation, then the resulting oligonucleotide generated by further addition to that subpopulation will be one base short. This will destroy the utility of the oligonucleotide as a component in DNA which is to be expressed in recombinant culture because the deletion will shift the DNA out of reading frame, and it is difficult to detect contaminating deletion mutants in long oligonucleotide products. Thus, when preparing oligonucleotides for use in molecular biology it is important to select a dehydrating agent that exhibits high fidelity in chain elongation, i.e., high yield addition at each cycle. Selecting such superior agents other than those specifically set forth herein is accomplished by the use of a screening assay.

The screening assay to be employed is described in more detail in Example 2. A nucleoside H-phosphonate (N) is condensed to a carrier-bound nucleoside through four cycles, the last one of which should yield N_5, then the polynucleotide H-phosphonates are separated from the carrier, converted to the oligonucleotides and separated on HPLC in order to determine the distribution of oligomers from N_2 to N_5. If the selected dehydrating agent produces an oligomer mixture having greater than about 80 mole % of N_5 as measured by spectrophotometric adsorption corrected for the extinction coefficients of the oligomers, and usually greater than about 90%, then the agent will be considered preferred for the purpose of this invention. Alternatively, the dehydrating agent that is selected is one which, after 49 cycles of step d) followed by steps e) and f), yields an oligonucleotide composition wherein the desired 50 nucleotide oligonucleotide in the composition is present in a greater molar amount than any other oligonucleotide species in the composition. The agents used by the art for the preparation of dinucleosides have been phosphorylating or sulfonylating agents. Agents of these classes used by the art are not capable of meeting the rigorous standards herein, particularly diphenyl chlorophosphate, 2,4,6-triisopropylbenzenesulfonyl chloride or 2,4,6-triisopropylbenzene sulfonyl tetrazolide.

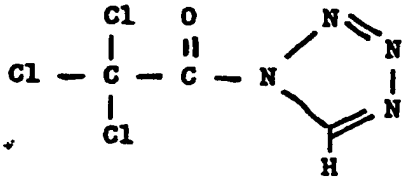
The preferred activating agents are acylating agents. Particularly preferred are acylating agents having the structure

wherein A is aryl or

in which the R groups are inert substituents preferably selected from the same or different normal or branched alkyl, aryl, halogen, heteroalkyl or heteroaryl, and X is a halogen or a pseudo-halogen, e.g. a heterocyclic

secondary amine or group capable of forming an active ester. R is preferably alkyl, X is preferably chloride or bromide. Pivaloyl chloride, wherein R is CH_3 and X is Cl, is the preferred acylating agent.

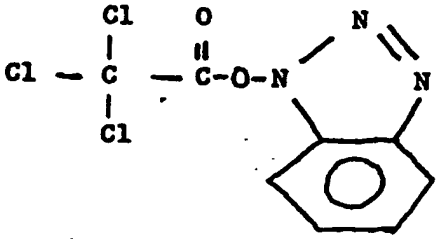
A representative heterocyclic amine X group is tetrazole. A typical example of this species is



EMI PA=7 FR=2 HE=30 WI=60 TI=CHE

It is produced in accord with methods known per se wherein $Cl_3C-\overset{O}{\overset{\|}{C}}-Cl$ is reacted with tetrazole.

A representative active ester is



produced by the reaction of $Cl_3C-\overset{O}{\overset{\|}{C}}-Cl$ with hydroxybenztriazole. Other suitable leaving groups will be apparent to the artisan.

The dehydrating agent typically is employed at a concentration of about 25 to 100 mM. Pivaloyl chloride preferably is present in a concentration of about 50 mM or 5 molar equivalents of pivaloyl chloride relative to the phosphonate monomer. The use of 10 equivalents reduced the yield of oligonucleotide, thus indicating that competitive acylation is yield limiting and that no extraneous capping step is required. The pivaloyl chloride reacts with the small proportion of aborted chains to produce a pivolate ester that is incapable of participating in further condensations. Thus, incomplete chains are capped without any need to introduce a separate step or additional reagents.

The quantity of phosphonate required in the condensation generally ranges about from 5 mM to 20 mM, ordinarily about 10 mM (~5eq. relative to the 5'-OH component). This is considerably less than the amount of monomer employed in known oligonucleotide synthetic processes, which typically require about 50 mM.

The solvent for the condensation reaction is an anhydrous organic solvent, preferably anhydrous pyridine/acetonitrile in volume proportions of 1:1.

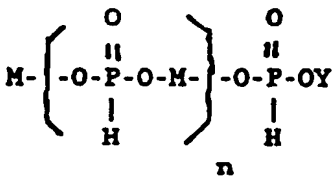
The reaction generally is conducted by mixing dehydrating agent and selected phosphonate solutions and then contacting the mixture with the carrier-bound oligonucleotide chain. Typically, the carrier is particulate and packed into a column over which the solution of monomer and dehydrating agent then is passed. The contact time generally is about from 0.5 to 5 minutes, with about 1.5 minutes being preferred. The reaction temperature is about from 10°C to 30°C, preferably 20°C.

The solution containing the residual dehydrating agent and nucleoside H-phosphonate is removed from the carrier after each cycle by washing with an organic solvent such as acetonitrile. Thereafter, the protecting group is removed from the added nucleoside, preferably by treatment with a 2.5% vol/vol dichloroacetic acid/CH_2Cl_2 solution, although 1% w/v trichloroacetic acid/CH_2Cl_2 or ZnBr-saturated nitromethane also are useful. Other deprotection procedures suitable for other known protecting groups will be apparent to the ordinary artisan. Then the carrier preferably is washed with anhydrous pyridine/acetonitrile (1/1, vol/vol) and the condensation reaction is repeated in as many additional cycles as are required to prepare the predetermined oligonucleotide.

After the required number of synthetic cycles the polynucleotide H-phosphonate is oxidized to the nucleic acid. In order to prepare oligonucleotides having phosphoramidate internucleotide linkages, the oxidation is conducted in the presence of a primary or secondary amine and, preferably, carbon tetrachloride. The preferred oxidizing agent is aqueous iodine to generate phosphate diesters into nucleotide linkages. Other representative oxidizing agents include N-chlorosuccinimide, N-bromosuccinimide, or salts of periodic acid. Thereafter, the oligonucleotide is separated from the carrier, in the preferred instance by incubation with concentrated ammonium hydroxide followed by filtration to remove the residual silica gel and evaporation of the oligonucleotide-containing solution. If desired the oligonucleotide then is purified by HPLC, polyacrylamide gel electrophoresis or other conventional techniques and the fidelity of the product confirmed by nucleic acid sequence analysis.

While the foregoing discussion has revolved around the consecutive addition of mononucleoside

phosphonates it will be understood that more than one nucleoside can be added in a given cycle by using poly (nucleoside phosphonates), generally di- or tri nucleoside phosphonates. These reagents are readily prepared by condensing a soluble nucleoside (which may or may not be bound to a carrier) to a nucleoside phosphonate otherwise as described above for chain initiation. Such dinucleoside diphosphonates are then employed in place of the mononucleoside phosphonates, or to prepare other poly(nucleoside phosphonates). These novel intermediates have the structure



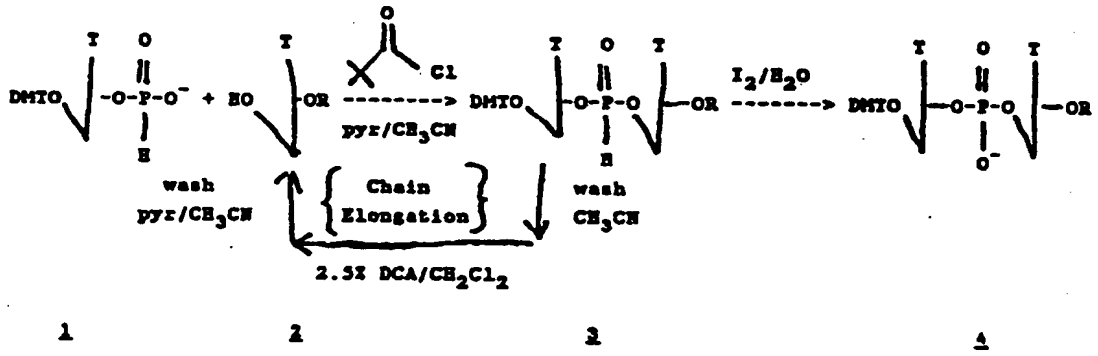
wherein Y is a counterion such as DBUH+, n is 1 or greater than 1, ordinarily 2, and M is a nucleoside. Ordinarily the nucleoside will be protected. An example is 5' Dimethoxytrityl-3'-thymidyl-5'-H-phosphonate-3'-thymidine-H-phosphonate. These agents should be purified and stored as anhydrous solids.

The following examples are illustrative of the invention but are not to be considered as limitations on its scope, which is defined by the appended claims.

EXAMPLE 1

Preparation of Eicosathymidylic Acid (T20) and Tetracontathymidylic Acid (T40)

The synthetic utility of 5'-dimethoxytrityl-3'-thymidine-H-phosphonate 1 was demonstrated by the synthesis of T_{20} and T_{40} on silica gel. 5'-Dimethoxytrityl-3'-trhymidine H-phosphonate was prepared as previously described and characterized by ^{31}P NMR (δ-0.27 ppm, J (P-H) = 605 Hz) (^{31}P NMR spectra were obtained in an anhydrous Pyr/CH_3CN (1/1) solution and chemical shifts are reported relative to 5 percent phosphoric acid/D_2O (external standard). The reaction scheme (scheme 1) was as follows, wherein DMT is dimethoxytrityl, pyr is pyridine, DCA is dichloroacetic acid and R is succinyl silica.



The polymer support (silica gel) was derivatized to 25μmole 3'-succinyl thymidine per gram of silica gel as previously described (Chow et al., Id). The synthetic cycle consisted of a condensation reaction of 2 with 10mM 1 (~5eq. relative to 5'-OH component 2) and 50 mM pivaloyl chloride at 20°C in anhydrous pyridine/acetonitrile (1/1) for 5 min, followed by dimethoxytrityl deprotection of 3 using 2.5% DCA/CH_2Cl_2 (2 min). After the required number of synthetic cycles, the polythymidine H-phosphonate product 3 was oxidized to polythymidylic acid 4 with 0.2M I_2 in tetrahydrofuran/Pyr/H_2O (90/5/5) (5 min). The product 4 was removed from the solid support (conc. NH_4OH/5 hr./55°C) and evaporated. HPLC analysis of the crude product T_{20} demonstrated that the product peak coeluted with a T_{20} standard prepared by the phosphoramidite method. The T_{20} and T_{40} crude products also were evaluated by polyacrylamide gel electrophoresis and visualized by U.V. shadowing. The analytical data clearly demonstrated the high yield synthesis of both T_{20} and T_{40}. T_{40} was further evaluated by 5'-end labeling with γ-^{32}P-ATP using polynucleotide T4 kinase followed by complete degradation with snake venom phosphodiesterase. By all criteria, the synthetic T_{40} was comparable to a standard T_{40}.

EXAMPL

Identification of Preferred Dehydrating Agent

The method of Example 1 was repeated except that parallel cycles were run with diphenyl chlorophosphate (DPCP) as the dehydrating agent and the reaction was only continued through 4 cycles to pentathymidylic acid (T_5). The crude column eluate was subjected to HPLC analysis to determine the percentage of oligonucleotides representing premature chain terminations. The HPLC resin was the Zorbax-NH_2 brand. The flow rate was 2.5 ml/min. of a linear gradient of 25mM to 250 mM KH_2PO_4 in 15% vol/vol CH_3CN (pH 5.5). Fractions were collected over 15 min. and adsorbance measured at 254 nm. The heights of the single, substantially symmetrical peaks for each of T_2, T_3, T_4 and T_5 were taken from the HPLC charts and converted into percentages to total polythymidine in each crude eluate, correcting for the coefficient of extinction for each oligomer (by multiplying the peak values by 2.5, 1.75, 1.25, and 1 respectively). The results are shown in the following table.

oligomer	Percent oligomer	
	DPDC	Pivaloyl chloride
T2	25	2.5
T3	23	3.5
T4	44	5
T5	8	89

These results demonstrate that pivaloyl chloride is a preferred dehydrating agent, producing only about 11% of prematurely terminated oligomers through 4 cycles.

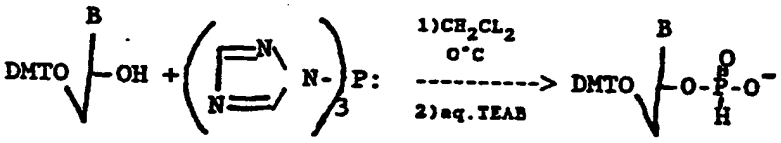
The yield of T5 using DPDC was only about 10% of that obtained by the use of pivaloyl chloride. Aryl sulfonyl chlorides and carbodiimides produced no detectable T5. Thus the method of this invention using the preferred acylating agents unexpectedly results in greater yields and reduced premature chain termination.

EXAMPLE 3

Preparation of Nucleoside H-Phosphonate

The following scheme was used to prepare deoxynucleosides for use herein.

Scheme 2



I II 1

B – a) thymine
b) N^6-benzoyl adenine
c) N^4-benzoyl cytosine
d) N^2-isobutyryl guanine
e) N^6-diisobutyl formamidine adenine

To a stirred solution of PCl_3 (75mmole) and N-methyl morpholine (750mmole) in 750 ml anhydrous CH_2Cl_2 was added 1,2,4-triazole (250mmole) at room temperature. After 30 min. the reaction mixture was cooled at 0°C and 5'-Dimethoxytrityl thymidine (Ia, 15mmole, dried by co-evaporation from CH_3CN) in 200 ml anhydrous CH_2Cl_2 was added dropwise over 20 min., stirred for 10 min., poured into 600 ml of 1.0M aqueous triethylammonium bicarbonate (TEAB, pH 8.5), shaken and separated. The aqueous phase was extracted with 200 ml CH_2Cl_2 and the combined organic phase was dried over Na_2SO_4 and evaporated to a foam. Silica gel column chromatography (2 percent ET_3N/CH_2Cl_2-> 2 percent ET_3N/10 percent $MeOH/CH_2Cl_2$) followed by TEAB extraction and evaporation yielded 5'-dimethoxytrityl-3'-thymidine H-phosphonate (Ia) in 90 percent yield. All other deoxynucleoside H-phosphonates were prepared by the same procedure. Table 1 reports isolated yields and ^{31}PNMR data:

Table 1

Nucleoside H-phosphonate	^{31}P NMR[1] Chemical Shift	J(P-H)	Yield[2] (Percent)
thymidine (1a)	-0.3 ppm	605 Hz	90
deoxyadenosine (1b)	-0.3 ppm	600 Hz	85
deoxycytidine (1c)	-0.3 ppm	603 Hz	86
deoxyguanosine (1d)	-0.6 ppm	605 Hz	77

1) ^{31}P NMR spectra were obtained in an anhydrous Pyr/CH_3CN (1/1) solution and chemical shifts are reported relative to 5 percent phosphoric acid/D_2O (external standard).
2) Isolated yield based upon protected deoxynucleoside (Ia-d).

EXAMPLE 4

Synthesis of Deoxyoligonucleotides

This currently represents the best mode for practicing the invention. Syntheses were performed with a Biosearch Model 8600 DNA synthesizer. Trimethylacetyl chloride (pivaloyl chloride) was distilled at atmospheric pressure and stored under Argon (Ar). Pyridine (Pyr) was distilled from p-toluenesulfonyl chloride, then freshly distilled from calcium hydride. Acetonitrile (CH_3CN) was dried over activated 3 Angstrom molecular sieves. Deoxy-nucleoside H-phosphonates (1a-d) were dried by co-evaporation from anhydrous CH_3CN and reconstituted in anhydrous Pyr/CH_3CN (1/1). Syntheses were performed on control pore glass (0.1 µmole scale) using the following protocol.

Synthetic Cycle (Scheme 1, Example 1)

1) Wash - anhydrous CH_3CN (45 sec).
2) Deblock - 2.5 percent dichloroacetic acid (DCA)/CH_2Cl_2 (1 min.).
3) Wash - anhydrous Pyr/CH_3CN (45 sec).
4) Couple - 10 mM deoxynucleoside H-phosphonate (1a-d), 50 mM pivaloyl chloride in anhydrous Pyr/CH_3CN (1/1 by vol) (1.5 min).
5) Repeat steps 1-4 until the nucleotide sequence is complete.
6) Deblock - 2.5 percent DCA/CH_2Cl_2 (1 min).
7) Oxidize H-phosphonate DNA with: 1) 0.1 M I_2 in Pyr/NMI/H_2O/THF (5/1/5/90 by vol) (2.5 min), 2)0.1 M I_2 in Et_3N/H_2O/THF (5/5/90 by vol) (2.5 min). The DNA was removed from the polymer, deprotected (conc. NH_4 OH, 55°C, 5 hrs.) and evaporated.

Deoxynucleoside H-phosphonates (1a-d) have been used for the complete synthesis of deoxyoligonucleotides (DNA) up to 107 bases in length, although this is not an upper limit on synthetic capability. The synthetic protocol described above uses a minimal amount of deoxynucleoside H-phosphonate (2.5 mg/coupling) in a simplified and rapid procedure (4 min/cycle). The internucleoside phosphorus is protected from unwanted side reactions by its oxidation state and the native phosphate diester is generated after synthesis by aqueous I_2 oxidation.

Oxidation of a polymer bound polynucleoside H-phosphonate (3) with 0.1M I_2 in a Pyr/H_2O/THF (5/5/90) solution has been inadequate for consistent results. The aqueous I_2 oxidation of dialkyl H-phosphonates is subject to general base catalysis (Lewis, E.S. and Spears, L.G., J. Amer. Chem. Soc., 107, 3918 (1985)) and we have determined that addition of N-methyl imidazole (NMI) or stronger bases (i.e. N-methyl morpholine or triethylamine (Et_3N) increases the rate of oxidation and is essential for the oxidation of long deoxyoligonucleoside H-phosphonates (3, >40 bases). Dialkyl H-phosphonates are susceptible to alkaline hydrolysis (Kume, A., Fujii, M., Sekine, M. and Hata, T., J. Org. Chem., 49, 2139 [1984]) and we have observed that the aqueous oxidation of polynucleoside H-phosphates (3) using Et_3N is complicated by competitive hydrolysis. The I_2 oxidation of long deoxyoligonucleoside H-phosphonates (3, >40 bases) first with a weakly

basic sol[redacted](Pyr/NMI) followed by oxidation with a strongly basic[redacted] solution (Et_3N) gives the highest yield of product deoxyoligonucleotide (4). Additionally we have found that an aqueous I_2 solution disproportionates to IO_3^- and I^- (Lewis, Id.) and an alkaline IO_3^- solution will not oxidize a dinucleoside H-phosphonate (3) (Brown, D.M. and Hammond, P.R., J. Chem. Soc., 4229 [1960]). Therefore it is necessary to prepare separate solutions [A = 0.2M I_2/THF, B = Base/H_2O/THF (1/1/8)] and mix them immediately prior to use for the consistent and rapid oxidation of deoxyoligonucleoside H-phosphonates (3).

Experiments were performed to evaluate the stability of the deoxyoligonucleoside H-phosphonate linkage (3) to the reagents used in deoxyoligonucleotide synthesis. A 24-mer was prepared and, prior to oxidation of the deoxynucleoside-H-phosphonate (3), aliquots of the solid support were treated for 4 hours with: 1) anhydrous Pyr/CH_3CN (1/1), 2) anhydrous CH_3CN, 3) 100 mM pivaloyl chloride in anhydrous Pyr/CH_3CN (1/1) and 4) 2.5 percent DCA/CH_2Cl_2, washed with CH_3CN, oxidized, washed, deprotected (conc. NH_4OH/5 hr./55°C) and evaporated. After 5'-end labeling with γ-^{32}P-ATP using polynucleotide T4 kinase the samples were analyzed by gel electrophoresis and autoradiography (data not shown). Under these conditions no detectable degradation of the polynucleoside H-phosphonate (3) is observed, but addition of 1 percent H_2O to CH_3CN or the Pyr/CH_3CN solution leads to detectable degradation in the 4 hr. time period. These results indicate that the H-phosphonate linkage serves as a phosphorus protecting group throughout synthesis.

It has been shown that 3' or 5'-phosphate triester stabilizes the glycosidic bond of deoxyadenosine to acid mediated depurination (Tanaka, T. and Letsinger, R.L., Nucl. Acids Res., 10, 3249 (1982)). The effect an internucleoside H-phosphonate linkage (3) would have on the rate of depurination was assessed. A polymer bound 3'-thymidine-5'-deoxyadenosine dinucleoside H-phosphonate, protected with a N^6-benzoyl amide (3b,a) or N^6-diisobutyl formamidine (3e,a), was treated with 2.5 percent DCA/CH_2Cl_2 for 24 hours and analyzed. No detectable difference in rate of depurination between the H-phosphonate diester (3) and the methyl phosphate triester was observed (data not shown). This experiment also confirmed the added stability of the N^6-amidine protected deoxyadenosine relative to the N^6-benzoyl amide protected deoxyadenosine.

Specific sequences of DNA were synthesized using the described protocol and the crude product characterized.

Table

Sequences of synthesized deoxyoligonucleotides

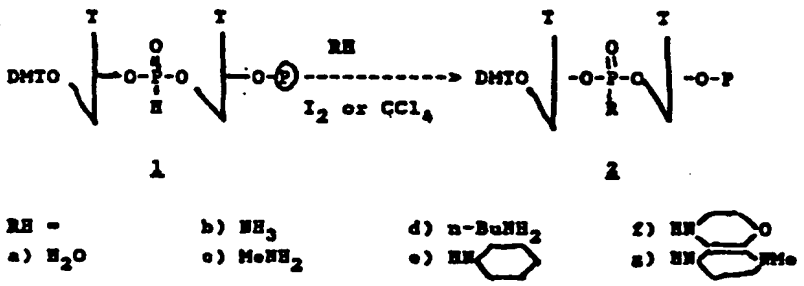
```
                                    *1                  *2
78a   5'-AGCTCTCGTAAAAAGGGTATCGACAATGAAAGCAATTTTCGTACTGAAAGGTTCACTGGA
78b   3'-    GAGCATTTTTCCCATAGCTGTTACTTTCGTTAAAAGCATGACTTTCCAAGTGACCT

          *3
      CGAATTCTGATTGAATGA    -3'
      GCTTAAGACTAACTTACTCTAG-5'

99    5'-AGTAGCAAGCTTGAGGTGTGGCAGGCTTGAGATCTGGCCATACACTTGAGTGACAATGAC
      ATCCACTTTGCCTTTCTCTCCACAGGTGTCCACTCCCAG-3'

107a  5'-AATTCATGAAAAAGAATATCGCATTTCTTCTTGCATCTATGTTCGTTTTTTCTATTGCTA
107b  3'-    GTACTTTTTCTTATAGCGTAAAGAAGAACGTAGATACAAGCAAAAAAGATAACGAT

      CAAACGCGTATGCAGACTCATGGATGGAGGAAGTTATTAAATTATGC    -3'
      GTTTGCGCATACGTCTGAGTACCTACCTCCTTCAATAATTTAATACGCCGG-5'
```

The deoxyoligonucleotides 78a, 78b, and 99 were also synthesized with methoxy diisopropylamino phosphoramidites and are included in the Table for comparison. The amidite syntheses were carried out using a standard protocol except that a 30 second aqueous wash was incorporated to oxidation to hydrolyze possible phosphite-heterocyclic base adducts. The data presented in the Table clearly demonstrate the high yield synthesis of product deoxyoligonucleotides using deoxynucleoside H-phosphonate intermediates. At this time the sequence fidelity of H-phosphonate DNA is lower than that produced by phosphoramidites; however, this disadvantage is offset by a simpler synthetic cycle and significantly lower concentration of deoxynucleoside H-phosphonate required for synthesis.

EXAMPLE 5

The dinucleotide phosphoramidates 2e and 2f (Scheme 3) were initially prepared by oxidation of the polymer bound dinucleoside H-phosphonate 1

Scheme 3



with 0.05M I_2 in a solution of the corresponding amine (10 percent) and tetrahydrofuran (THF) for 5 min. The product amidates were removed from the solid support (50 percent conc. ammonium hydroxide (NH_4OH)/Dioxane, 8 hrs., r.t.) and evaporated. ^{31}P NMR spectra of both products show the presence of approximately equal amounts of diasteriormers (^{31}P NMR spectra were obtained in $CDCl_3$ and chemical shifts are reported relative to 5 percent phosphoric acid/D_2O (ext. std.); 2e δ-9.1, -8.8ppm; 2f δ -7.9, -7.6 ppm.). The oxidation of H-phosphonate diesters to phosphoramidates is complicated by competitive hydrolysis, generating the phosphate diester 2a. Carbon tetrachloride (CCl_4) oxidation of H-phosphonate diesters to phosphoramidates (Atherton et al., J. Chem. Soc. 660 (1945), Zwierzak, A., Synthesis 507 (1975)) offers the advantage of low water solubility into CCl_4. All dinucleotide phosphoramidates (2b-g) were prepared with a 10 percent solution of the corresponding amine in CCl_4 (5 min) (A saturated solution of ammonia or methyl amine in Dioxane/CCl_4 (1/4) was used to prepare the corresponding phosphoramidates). High performance liquid chromatography (HPLC) of the samples indicate a high yield of product phosphoramidate with very little competitive hydrolysis (<3 percent dithymidine phosphate diester, 2a).

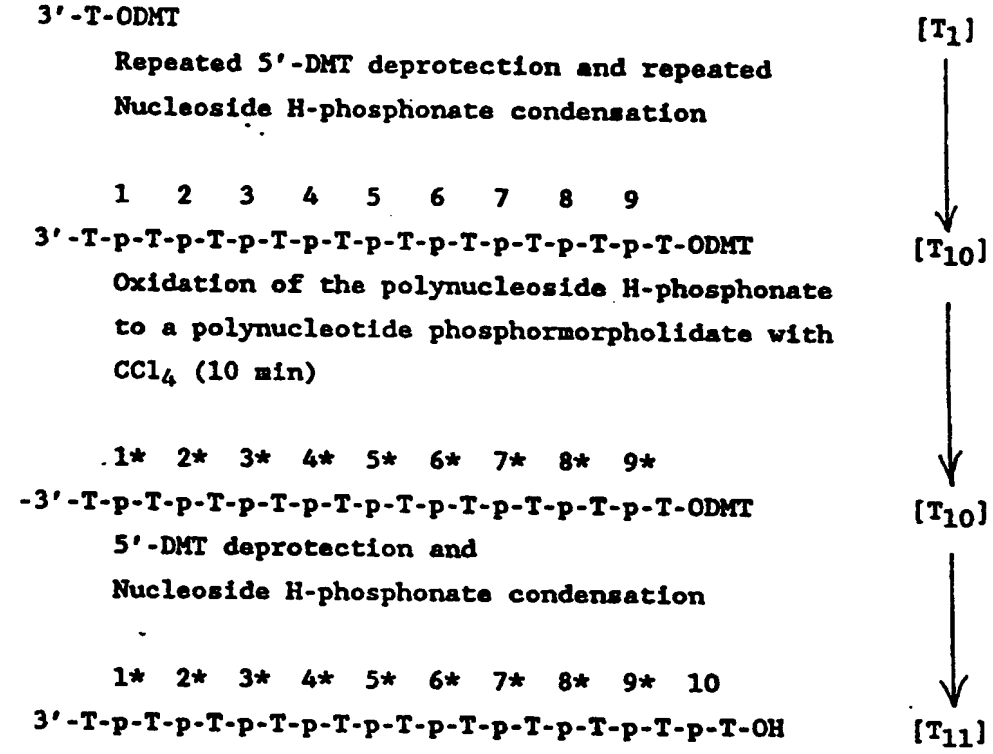
The stability of the phosphoramidate linkage to conc. NH_4OH was examined for each and the results indicate the phosphoramidates 2c-g are stable to the conditions necessary for removal of the common heterocyclic N-acyl protecting groups (55°C, 5 hrs). The phosphoramidate 2b rapidly decomposes under these conditions ($t_{1/2}$-15 min) to a mixture of the 3' and 5' phosphoramic acid monoesters (Tomasz, J., Nucleosides and Nucleotides 2, 51 (1983). The stability of the dinucleotide phosphoramidates (2b-g) to enzymatic degradation by exonucleases was examined using spleen phosphodiesterase and snake venom phosphodiesterase. Incubation of the dinucleotides (2a-g) with the corresponding enzyme/buffer solution at 37°C for 1 hr led to complete degradation of the phosphate diester (2a with no detectable degradation of the phosphoramidates 2b-g (assessed via reverse phase HPLC). The observed enzymatic stability of the dinucleotide phosphoramidate 2b is contrary to the claims of Ogilvie (Nemer et al., Tetrahedron Lett. 21, 4149 (1980)) but is in agreement with those reported by Letsinger (Letsinger et al., Nucl. Acids Res. 14, 3487 (1986)).

To further explore the scope of this oxidation procedure, undecathymidylic acid (T_{11}) was prepared containing from 1 to 9 phosphormorpholidate linkages. The synthesis of polythymidine H-phosphonate on controlled pore glass was carried out using the synthetic protocol described above. The strategy outlined in Scheme 4 demonstrates the synthesis of T_{11} containing nine phosphormorpholidate linkages followed by one phosphate diester linkage. This strategy was used for all T_{11} products containing 3' phosphoramidate linkages.

SCHEME 4

Synthesis of T_{11} containing nine phosphormorpholidate linkages (3' end) followed by one phosphate diester linkage (5' end).

* indicates phosphormorpholidate linkage



The products were removed from the solid support with conc. NH_4OH (2hr/r.t.) and evaporated. Polyacrylamide gel electrophoresis (PAGE) and U.V. shadowing indicated a major product band with each. The band was cut, crushed, eluted with H_2O and isolated by reverse phase HPLC (C_{18}). An autoradiogram was derived from 5'-end labeling of these products with T4 polynucleotide kinase and γ-^{32}P ATP followed by PAGE (17 percent polyacrylamide/7M urea). The change in electrophoretic mobility of the different T_{11} products was very pronounced, each additional morpholidate resulting in an increase in mass and a decrease in charge. It is interesting to note that the electrophoretic mobilities of the different T_{11} products do not follow a strict mass/charge ratio dependence. The products containing many phosphoramidate linkages (high mass/charge ratio) have greater mobility than would be predicted based upon the mobilities of the T_{11} products with fewer phosphoramidates. Treatment of each of these polythymidine phosphormorpholidates with 85 percent formic acid (95°C, 15 min) generated T_{11} containing all diester linkages.

Nucleoside H-phosphonate diesters have been used as precursors to thiophosphate analogues (Fujii et al., Tetrahedron Lett. 26, 935 (1986)).This conversion is accomplished in high yield (>98 percent) directly from the polymer bound oligonucleoside H-phosphonate diester (1) by treatment with a solution of 0.1M S_8 in Triethylamine (TEA)/carbon disulfide (1/9, 5 min). In order to prepare radioisotopically labelled oligonucleotides having extremely high specific activity for use as hybridization probes, the S_8 that is employed will contain a proportion of ^{35}S radioisotope.

Triesters of oxygen are prepared by reaction of oligonucleoside H-phosphonates (1) with a 10 percent solution of the corresponding alkanol (ROH) in N-methyl imidazole/TEA/CCl_4 (5/5/90). R is aryl, alkyl, heteroalkyl or heteroaryl generally having less than 10 carbon atoms, preferably MeOH or n-BuOH. This reaction is very susceptible to competitive hydrolysis and care must be taken to assure anhydrous conditions.

The primary and secondary amines b) - g) are to be considered merely representative. It is within the scope herein to employ substituents having the general formula - N(Y) - X wherein X and Y are the same or different and are selected from the group of hydrogen, alkyl, heteroalkyl, heteroaryl or aryl, or are taken together to form a cycloalkene, cycloalkane or heterocycle. The species in which X or Y are, or are conjugated to form heteroalkyl and heteroaryl substituents are preferred for use as in vitro hybridization probes, whereas the alkyl or aryl substituents are preferred for in vivo hybridization.

The heteroatom in heteroaryl ROH, X or Y is selected from the group of S, N or O, about from 1 to 5 of which are present in the heteroaryl group. Heteroaryl species that are particularly useful in in vitro diagnostics are those in which the heteroaryl group is fluorescent, for example, rare earth metal chelates for use in pulsed fluorescence, or other known substituents for use in polarized fluorescence hybridization assays. Such fluorescent labelled oligonucleotides are particularly useful in hybridization assays because it is possible to conduct the assays without a phase separation by detecting changes in fluorescence upon hybridization to the target DNA, e.g. changes in polarization, emission wavelength, absorption wavelength, emission intensity and the like. The method herein facilitates the preparation of oligonucleotides which are heavily substituted with such fluorescent species and accordingly exhibit high specific activity.

The results presented above demonstrate that polynucleoside H-phosphonates are valuable precursors to a variety of internucleotide phosphate analogues. Oxidation of a polynucleoside H-phosphonate in the presence of amines leads to the corresponding polynucleotide phosphoramidate in high yield. The H-phosphonate method of DNA synthesis is a simple, fast and reagent efficient procedure that can now be applied to the rapid synthesis of DNA containing internucleotide phosphate analogues. This method provides an easy and efficient route to a variety of internucleotide phosphate analogue of DNA containing the naturally occurring nucleotides.

Claims

1. A method for the synthesis of a polynucleotide comprising (a) providing a nucleoside bound to a carrier (b) condensing a blocked nucleoside H-phosphonate with the 5' hydroxyl of the carrier-bound nucleoside in the presence of a dehydrating agent, (c) removing the 5' blocking group of the nucleoside H-phosphonate of step b) from the carrier, (d) sequentially repeating steps b) and c) using nucleoside H-phosphonates until a polynucleotide H-phosphonate having greater than two nucleotides has been obtained, (e) oxidizing the polynucleotide H-phosphonate to form the corresponding polynucleotide, and (f) separating the polynucleotide from the carrier.

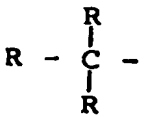
2. The method of claim 1 wherein the dehydrating agent is an acylating or phosphorylating agent.

3. The method of claim 2 wherein the dehydrating agent is other than an aryl sulfonyl chloride or aryl sulfonyl tetrazolide, diphenyl chlorophosphate, or a carbodiimide.

4. A method of claim 2 wherein the dehydrating agent is an acylating agent.

5. The method of claim 4 wherein the acylating agent has the structure

wherein A is aryl, heteroaryl or



in which R is the same or different normal or branched alkyl, heteroalkyl, aryl, heteroaryl or halogen and X is halogen or a pseudohalogen selected from the group of azide, heterocyclic secondary amines or groups capable of forming active esters.

6. The method of claim 5 wherein X is chloride.

7. The method of claim 5 wherein the acylating agent is pivaloyl chloride.

8. The method according to any one of the preceding claims wherein the dehydrating agent results in premature chain termination in less than about 20 mole % of the total oligomer population after four cycles.

9. The method of any preceding claim wherein the dehydrating agent is an organic acid halide.

10. The method according to any preceding claim wherein the nucleoside is a deoxyribonucleoside.

11. The method according to any preceding claim wherein steps b) and c) are repeated through at least 2 cycles.

12. The method according to claim 11 wherein steps b) and c) are repeated through about from 4 to 125 cycles.

13. The method according to any preceding claim wherein the dehydrating agent is an acid chloride.

14. The method according to any preceding claim wherein the dehydrating agent is capable of capping aborted oligonucleotide phosphonate chains.

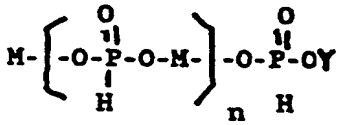
15. A method according to any preceding claim wherein the step e) occurs only after the entire polynucleotide H-phosphonate has been synthesized.

16. The method according to any preceding claim which is such that if 49 cycles of step d) are followed by steps e) and f), a 50 nucleotide polynucleotide is present in a greater amount than any other polynucleotide separated from the carrier.

17. The method according to any preceding claim wherein the nucleoside H-phosphonates are predetermined.

18. The method of claim 17 which is such that if 4 cycles of step d) are followed by steps e) and f), greater than about 80 mole percent of the polynucleotides separated from the carrier consists of a polynucleotide containing 5 predetermined nucleotides.

19. A composition having the formula



wherein Y is a counterion, n is ≥ 1, and M is a nucleoside.

20. The composition of claim 19 wherein M is a protected nucleoside.

21. The composition of claim 19 wherein the protecting group is a dialkyl formamidine.

22. A method for the preparation of internucleotide phosphate analogues of polynucleotides, comprising

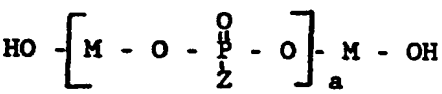
a) providing a carrier-bound polynucleoside H-phosphonate containing greater than two nucleotides,

b) reacting the polynucleoside H-phosphonate with an agent selected from i) S_8, ii) ROH wherein R is alkyl, heteroalkyl, heteroaryl or aryl, or iii) H-$\underset{\underset{Y}{|}}{N}$-X wherein X and Y are the same or different and are hydrogen, aryl, heteroaryl, heteroalkyl, alkyl, or are taken together to form a cycloalkene, cycloalkane or heterocycle, and

c) recovering the internucleotide phosphate analogue.

23. The method of claim 22 wherein ROH or H-$\underset{\underset{Y}{|}}{N}$-X are fluorescent.

24. A composition comprising a polynucleotide with at least one substituted internucleotide phosphate, said polynucleotide having the formula



wherein

1) -Z is -OH, -SH, -O-R, or -$\underset{\underset{Y}{|}}{N}$-X and a is an integer greater than about 10;

2) at least 1 Z is not -OH;

3) R is aryl, heteroaryl, alkyl or heteroalkyl;

4) X or Y are hydrogen, heteroalkyl, alkyl, aryl or heteroaryl, or are taken together to form a heterocycle, heterocyclic alkane or alkene; and

5) M is a nucleoside.

25. The composition of claim 24 wherein X and Y are taken together to form an oxygen-containing heteroaryl substitutent.

26. The composition of claim 24 or 25 wherein -OR or -$\underset{\underset{Y}{|}}{N}$-X are fluorescent.